Antibiotic and Antimalarial Quinones from Fungus-Growing Ant-Associated Pseudonocardia sp.

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Antibiotic and Antimalarial Quinones from Fungus-Growing Ant-Associated *Pseudonocardia* sp.

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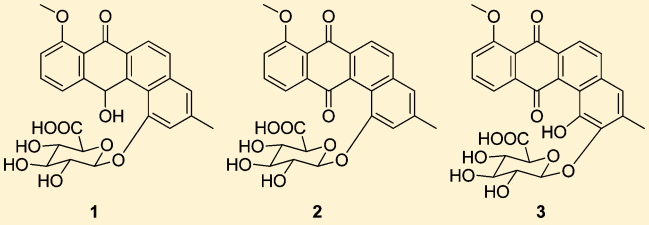
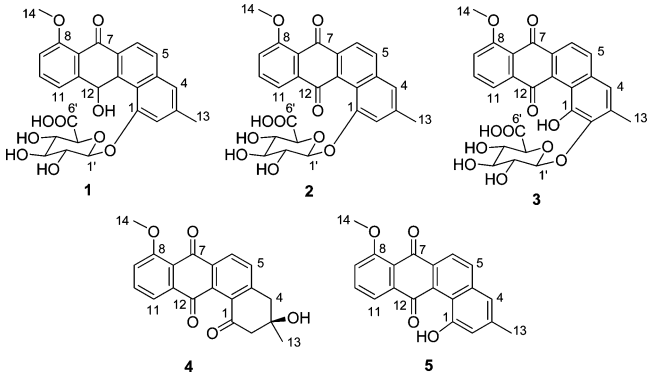
Supporting Information

ABSTRACT: Three new members of the angucycline class of antibiotics, pseudonocardones A−C (**1**−**3**), along with the known antibiotics 6-deoxy-8-*O*-methylrabelomycin (**4**) and X-14881 E (**5**) have been isolated from the culture of a *Pseudonocardia* strain associated with the fungus-growing ant *Apterostigma dentigerum*. Compounds **4** and **5** showed antibiotic activity against *Bacillus subtilis* 3610 and liver-stage *Plasmodium berghei*, while **1**−**3** were inactive or only weakly active in a variety of biological assays. Compound **5** also showed moderate cytotoxicity against HepG2 cells.



Bacteria of the genus *Pseudonocardia* associate with fungus-growing ants[1] and produce antibiotics that presumptively play a role in suppressing fungal pathogens in ants' gardens.[2,3] While *Pseudonocardia* belongs to the Actinomycetales, which are well known for their remarkable ability to produce bioactive secondary metabolites, relatively few natural products have been described from *Pseudonocardia* spp. Antibiotics discovered from *Pseudonocardia* spp. include dentigerumycin,[3] pseudonocardians A and B,[4] phenazostatin D,[5] and NPP.[6] Of these, only dentigerumycin was discovered from a *Pseudonocardia* strain associated with fungus-growing ants, and these symbionts likely have many more undiscovered natural products. As part of our ongoing effort to explore natural products from bacterial symbionts,[3,7−9] we have investigated the natural products from *Pseudonocardia* sp. EC080529-01, isolated from the cuticle of the fungus-growing ant *Apterostigma dentigerum*.[10] This strain produces three new members of the angucycline family of antibiotics,[11] pseudonocardones A (**1**), B (**2**), and C (**3**), along with the known antibiotics 6-deoxy-8-*O*-methylrabelomycin (**4**)[12] and X-14881 E (**5**).[13]



Pseudonocardia sp. EC080529-01 was grown on solid ISP-2 medium, and the agar was extracted with EtOAc followed by MeOH. HPLC-MS analysis of the EtOAc extract revealed two peaks with interesting UV spectra (λ_{max} at 381 and 410 nm, respectively). HPLC-MS analysis of the MeOH extract revealed three additional polar peaks with λ_{max} at 348 nm, 388, and 409 nm, respectively. Production cultures of *Pseudonocardia* sp. EC080529-01 were grown on solid ISP-2 medium and extracted with EtOAc followed by MeOH. The EtOAc extract was purified (see Experimental Section) to give 6-deoxy-8-*O*-methylrabelomycin (**4**, 8.3 mg) and X-14881 E (**5**, 3.5 mg). The MeOH extract was purified (see Experimental Section) to give pseudonocardones A (**1**, 2.0 mg), B (**2**, 0.9 mg), and C (**3**, 1.8 mg). The known compounds **4** and **5** were identified by 1D and 2D NMR spectroscopy, and their structures were confirmed by comparison of their spectroscopic data with the literature values.[12,13]

Pseudonocardone A (**1**) gave a peak in the HRESI(+) MS consistent with a molecular formula of $C_{26}H_{24}O_{10}$. The NMR data (Figures 1 and 2 and Table 1) obtained for **1** indicated that it was related to the angucycline family of antibiotics and that it was similar in structure to **5**. The peak in the UV spectrum of **5** at λ_{max} = 410 nm had shifted to λ_{max} = 348 nm. While compound **5** showed two carbon resonances typical of a quinone (δ_C 191.3 and 182.0), compound **1** showed only one carbon resonance in this range (δ_C 186.0). Instead, compound **1** showed a signal for an oxygenated methine resonance (δ_C 66.5; δ_H 6.93). This methine resonance showed HMBC correlations to C-6a (δ_C 132.5), C-7a (δ_C 120.8), C-11 (δ_C 123.8), C-11a (δ_C 147.7), C-12a (δ_C 139.6), and C-12b

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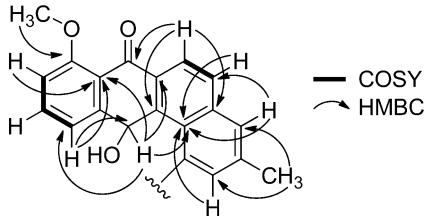

Figure 1. Key COSY and HMBC correlations in the aglycone moiety of pseudonocardone A (**1**).

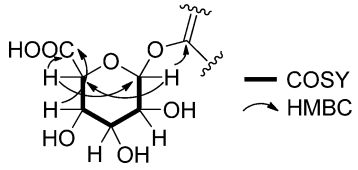

Figure 2. Key COSY and HMBC correlations in the sugar moiety of pseudonocardone A (**1**).

(121.6), indicating that C-12 is reduced in **1** to give a hydroquinone tautomer rather than a quinone as found in **5**.

Additional signals were present in the NMR spectra of **1** that could not be attributed to the angucycline core. Subtracting the atoms accounted for by the hydroquinone substructure showed that the remaining fragment had to account for $C_6H_9O_6$. A series of oxygenated methine resonances in the HSQC spectrum (δ_C 73.1−77.1 and δ_H 3.63−4.16) and a resonance typical of an anomeric carbon (δ_C 103.8 and δ_H 5.23) suggested that **1** was glycosylated. An HMBC correlation from the anomeric proton at δ_H 5.23 to δ_C 156.9 (C-1) showed that **1** was glycosylated at the oxygen atom attached to C-1. A series of COSY and HMBC correlations (Figure 2) revealed that the sugar was a hexose. Finally, HMBC correlations from δ_H 3.76 (H-4′) and 4.16 (H-5′) to a carbonyl carbon at δ_C 173.0 (C-6′) showed that the sugar had been oxidized to the carboxylic acid at C-6′.

The relative configuration of the sugar residue in **1** was determined by coupling constants and a NOESY experiment. Large coupling constants (J values ranged from 7.6 to 10.0 Hz; see Table 1) indicated that all of the protons must be axial, and therefore the sugar corresponds to β-glucuronic acid. This assignment was supported by NOESY correlations between δ_H 5.23 (H-1′) and δ_H 3.63 (H-3′), between δ_H 5.23 (H-1′) and δ_H 4.16 (H-5′), between δ_H 3.93 (H-2′) and δ_H 3.76 (H-4′), and between δ_H 3.63 (H-3′) and δ_H 4.16 (H-5′) (Figure 3). The absolute configuration of the sugar moiety was not determined. The absolute configuration of C-12 was also not determined.

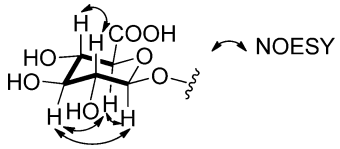

Figure 3. NOESY correlations in the sugar moiety of pseudonocardone A (**1**).

Pseudonocardone B (**2**) gave a peak in the HRESI(+) MS consistent with a molecular formula of $C_{26}H_{22}O_{10}$. The molecular formula of **2** differed from that of **1** by the loss of

Table 1. ^{1}H and ^{13}C NMR Data for Pseudonocardones A (1), B (2), and C (3) Recorded in CD_3OD at 600 MHz

	pseudonocardone A (**1**)		pseudonocardone B (**2**)		pseudonocardone C (**3**)	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	156.9		154.7			
2	114.7	7.21, s	115.8	7.25, s	144.7	
3	140.5		141.8		134.7	
4	124.0	7.37, s	122.9	7.45, s	120.3	6.95, s
4a	139.4		135.4		131.9	
5	130.0	7.78, d (8.8)	134.4	8.06, d (8.2)	133.0	8.97, d (9.4)
6	124.5	8.10, d (8.2)	123.0	8.16, d (8.8)	122.8	8.17, d (8.8)
6a	132.5		135.0		137.1	
7	186.0		182.8		183.1	
7a	120.8		120.8		119.9	
8	161.1		160.3		160.5	
9	112.8	7.13, d (8.2)	118.3	7.47, d (8.8)	119.3	7.49, d (8.2)
10	136.0	7.64, t (7.9)	136.7	7.78, t (7.9)	136.5	7.75, t (7.9)
11	123.8	7.34, d (8.6)	119.3	7.62, d (7.6)	121.1	7.78, d (7.0)
11a	147.7		139.9		138.4	
12	66.5	6.93, s	189.5		190.8	
12a	139.6		138.8		133.2	
12b	121.6		119.5		121.0	
13	21.8	2.47, s	21.6	2.52, s	17.0	2.50, s
14	49.4	3.95, s	56.4	4.01, s	56.6	3.99, s
1′	103.8	5.23, d (7.6)	101.8	5.26, d (7.0)	106.6	4.76, d (7.6)
2′	75.0	3.93, t (8.8)	74.5	3.58, m	75.1	3.71, dd (9.1, 7.9)
3′	77.1	3.63, t (9.4)	77.1	3.59, m	77.2	3.50, t (9.1)
4′	73.1	3.76, t (9.4)	72.6	3.60, m	72.9	3.63, t (9.4)
5′	76.7	4.16, d (10.0)	76.1	3.98, d (9.4)	76.8	3.55, d (10.0)
6′	173.0		172.6		173.5	

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two hydrogen atoms. The UV spectrum of **2** also differed significantly from that of **1**, with the low-energy λ_{max} having shifted from 348 nm in **1** to 388 nm in **2**. The NMR data obtained for **2** were very similar to that of **1**, suggesting that the compounds were closely related. The NMR signals corresponding to the C-12 oxygenated methine present in **1** were absent from the NMR spectra of **2**. Instead, the resonance at δ_H 7.62 (H-11) showed an HMBC to a carbon at δ_C 189.5 (C-12), typical of a quinone carbon, revealing that **2** is the quinone analogue of **1**.

Pseudonocardone C (**3**) gave a peak in the HRESI(+) MS consistent with a molecular formula of $C_{26}H_{22}O_{11}$. The molecular formula of **3** differed from the molecular formula of **2** by the addition of an oxygen atom. The UV and NMR data obtained for **3** were very similar to that of **2**, suggesting that they are closely related. The aromatic singlet present in **2** at δ_H 7.25 (H-2) was absent in the NMR spectrum of **3**, and the other aromatic singlet at δ_H 7.45 (H-4) was shifted upfield to δ_H 6.95 (H-4) in **3**. The methyl resonance at δ_H 2.50 showed an HMBC correlation to a downfield carbon at δ_C 144.7 (C-2), suggesting that C-2 was substituted with an oxygen atom. An HMBC correlation from the anomeric proton at δ_H 4.76 (H-1′) to C-2 showed that the sugar moiety was attached to C-2 instead of to C-1 as found in **1** and **2**. The lack of protons within three bonds from C-1 made the assignment of this position impossible to determine from the HMBC data. However, in order to satisfy the molecular formula of **3**, C-1 must be oxygenated as it is in **1**, **2**, and **5**.

Compounds **1**−**5** were tested for antibiotic activity against *Escherichia coli* K12, *Bacillus subtilis* 3610, *Candida albicans*, and *Saccharomyces cerevisiae*. Compounds **4** and **5** were active against *B. subtilis* 3610 with MIC values of 25 and 3.13 μg/mL, respectively. None of the compounds showed any activity against *E. coli*, *C. albicans*, or *S. cerevisiae* at concentrations as high as 50 μg/mL. Compounds **1**−**5** were also tested in a liver-stage malaria assay recently developed in one of our laboratories.[14] Compounds **4** and **5** were active against liver-stage *Plasmodium berghei* with IC_{50} values of 18.5 and 3.0 μM, respectively. Finally, compounds **1**−**5** were tested for cytotoxicity against HepG2 cells. Compound **5** was active against HepG2 cells with an IC_{50} value of 36.1 μM. The glycoside analogues (**1**−**3**) were completely inactive against *B. subtilis*, *E. coli*, *C. albicans*, *S. cerevisiae*, and HepG2 cells at concentrations as high as 50 μg/mL and showed only weak activity against liver-stage *P. berghei* with IC_{50} values of 38, 50, and >100 μM, respectively. The lack of activity for the glycosylated analogues provides insight into the structure−activity relationships of this family of compounds. A comparison of the activity of **2** with that of **5** shows that adding the glucuronic acid moiety at C-1 completely abolishes cytotoxic and antibiotic activity. Glycosylation of antibiotics has been proposed as one possible mechanism of self-resistance,[15,16] and this might explain the lack of biological activity observed for **1**−**3**.

EXPERIMENTAL SECTION

General Experimental Procedures. An Agilent 1200 Series HPLC system equipped with a diode array detector and a Phenomenex C_{18} column (5 μm, 250 × 21.2 mm) was used for preparative HPLC. For HPLC-MS analysis, an Agilent HPLC system equipped with a diode array detector and a 6130 Series quadrupole mass spectrometer was used with a Phenomenex C_{18} (5 μm, 100 × 4.6 mm) column. The following gradient was used for HPLC-MS analysis: 0−5 min, isocratic 10% CH_3CN + 0.1% formic acid; 5−25 min, linear gradient from 10% CH_3CN + 0.1% formic acid to 100% CH_3CN + 0.1% formic acid. NMR spectra were recorded in CD_3OD (for compounds **1**−**4**) or CD_2Cl_2 (for compound **5**) at 600 MHz and referenced to the internal solvent peak at δ_H 3.30 and δ_C 49.0 or δ_H 5.32 and δ_C 53.8, respectively. High-resolution mass spectrometry (HR-MS) was performed at the University of Illinois Urbana−Champaign Mass Spectrometry Facility.

Table 2. Cytotoxic Activities of 1−5 against HepG2 Cells and Antibiotic Activities of 1−5 against *P. berghei*, *E. coli*, *B. subtilis*, *C. albicans*, and *S. cerevisiae*[a]

	HepG2	*P. berghei*	*E. coli*	*B. subtilis*	*C. albicans*	*S. cerevisiae*
compound	IC_{50} (μM)	IC_{50} (μM)	MIC (μg/mL)	MIC (μg/mL)	MIC (μg/mL)	MIC (μg/mL)
1	>100	38	>50	>50	>50	>50
2	>100	50	>50	>50	>50	>50
3	>100	>100	>50	>50	>50	>50
4	>100	18.5	>50	25	>50	>50
5	36.1	3.0	>50	3.13	>50	>50

[a]Data represent the average of two experiments each performed in triplicate. The MIC is defined as the lowest concentration that gave less than 5% of the maximum growth.

Isolation of *Pseudonocardia* sp. EC080529-01. An ant colony of *A. dentigerum* was collected from Pipeline Road, Panama, on May 29, 2008, and placed in a sterile Petri dish with moist cotton. After allowing the nest to stabilize for a few days, the *Pseudonocardia* symbiont from this colony was isolated directly from the mesoternal lobe of a worker by scraping bacteria off the cuticle of the ant using a sterile scalpel and plating on chitin media following the methods of Caldera and Currie, and identified as *Pseudonocardia* sp. based on multilocus sequencing.[10]

Cultivation of *Pseudonocardia* sp. EC080529-01. Production cultures of *Pseudonocardia* sp. EC080529-01 were grown on solid ISP-2 medium (per liter: yeast extract, 4 g; malt extract, 10 g; glucose, 4 g) in 12 Petri plates (150 × 20 mm, 1.2 L total) for 7 d at 30 °C. The solid agar was cut into small cubes and soaked in EtOAc (1.2 L) overnight. The EtOAc was filtered and dried *in vacuo* to give the crude EtOAc extract. The solid agar was re-extracted overnight with MeOH (1.2 L), and the MeOH was filtered and dried *in vacuo* to give the crude MeOH extract. The crude EtOAc extract was dissolved in 90% MeOH−H_2O (20 mL) and passed through a C_{18} column, eluting with additional 90% MeOH−H_2O, in order to remove nonpolar components. The eluent from this column was diluted with H_2O to give a final MeOH concentration of 60%. This solution was passed through another C_{18} column and washed with additional 60% MeOH−H_2O solution, followed by 100% MeOH. The 60% MeOH−H_2O fraction was purified by preparative HPLC using the following gradient: 0−5 min, isocratic 20% CH_3CN−H_2O; 5−60 min, linear gradient from 20% CH_3CN−H_2O to 100% CH_3CN to give pure **4** (8.3 mg). The 100% MeOH fraction from this C_{18} column was purified by preparative HPLC using the following gradient: 0−10 min, isocratic 50% CH_3CN−H_2O; 10−60 min, linear gradient from 50% CH_3CN−H_2O to 100% CH_3CN to give pure **5** (3.5 mg). The crude MeOH extract was dissolved in H_2O and passed through an HP-20 column. The HP-20 column was washed with water to remove polar components, and the compounds of interest were then eluted with 100% MeOH. The 100% MeOH fraction was dried *in vacuo*, redissolved in 60% MeOH−H_2O, and passed through a C_{18} column to remove nonpolar components. The eluent from this column was diluted with H_2O to give a final MeOH concentration of 30%. This solution was passed through another C_{18} column and washed with additional 30% MeOH−H_2O, followed by 100% MeOH. The 100% MeOH fraction from this C_{18} column was purified by reversed-phase HPLC using the following gradient: 0−10 min, isocratic 10% CH_3CN−H_2O + 0.1% formic acid; 10−60 min linear gradient from

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10% CH_3CN–H_2O + 0.1% formic acid to 100% CH_3CN + 0.1% formic acid to give pure **1** (2.0 mg), **2** (0.9 mg), and **3** (1.8 mg).

*Pseudonocardone A (**1**):* colorless solid (2.0 mg); $[\alpha]^{20}_D$ −11 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 310 nm (3.11), 279 (sh), 271 nm (3.59); ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1; (+)-HRESI *m*/*z* 497.1438 $[M + H]^+$ (calcd for $C_{26}H_{25}O_{10}$, 497.1448).

*Pseudonocardone B (**2**):* yellow solid (0.9 mg); $[\alpha]^{20}_D$ −2 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 377 nm (3.52), 305 nm (3.87); ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD), see Table 1; (+)-HRESI *m*/*z* 495.1295 $[M + H]^+$ (calcd for $C_{26}H_{23}O_{10}$, 495.1291).

*Pseudonocardone C (**3**):* orange solid (1.8 mg); $[\alpha]^{20}_D$ −3 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 403 nm (3.53), 314 nm (3.96); ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (150 MHz, CD_3OD) see Table 1; (+)-HRESI *m*/*z* 511.1229 (calcd for $C_{26}H_{23}O_{11}$, 511.1240).

Antibiotic Assays. The appropriate test organism was grown in a 5 mL culture overnight in either LB medium (for *E. coli* and *B. subtilis*) or YPD medium (for *C. albicans* and *S. cerevisiae*) at 30 °C. In each case, the overnight culture was diluted with additional sterile medium (LB or YPD) to an OD_{600} of 0.01. Compounds **1**–**5** were dissolved in DMSO to give a concentration of 5 mg/mL and 2-fold serially diluted. These solutions (1 μL) were added to the wells of a 96-well plate, followed by the diluted culture of the test organism (99 μL) to give a final compound concentration ranging from 50 to 0.1 μg/mL. The cultures were allowed to grow for 24 h at 30 °C before the OD_{600} was measured using a plate reader. The MIC was defined as the lowest concentration that gave less than 5% of the maximum OD_{600}. Each antibiotic assay was performed in duplicate. Dynemicin A was used as a positive control and gave MIC values against *E. coli*, *B. subtilis*, *C. albicans*, and *S. cerevisiae* of 313, 0.16, 156, and 156 ng/mL, respectively.

Cytotoxic Assay. Compounds were tested for activity against HepG2 human hepatoma cells (ATCC) that were maintained in DMEM (Invitrogen), 10% FBS (Sigma), and 1% antibiotic–antimycotic (Invitrogen) in a standard tissue culture incubator (37 °C, 5% CO_2). For assays, compounds **1**–**5** (in DMSO) were added in triplicate to 15 000 cells in a 384-well plate. The final concentration of DMSO was 1%, and compounds varied from 0 to 50 μg/mL. Cells were incubated with the compounds for 2 days at 37 °C, and then liver cell viability was assessed with CellTiter-Glo (Promega). The relative signal intensity of each sample was evaluated with an EnVision (PerkinElmer) system.

Liver-Stage *P. berghei* Assay. Liver-stage *P. berghei* assays were performed using a luciferase-expressing sporozoite strain of *P. berghei* ANKA. Parasites were obtained from dissection of *Plasmodium*-infected *Anopheles stephensi* mosquitoes (New York University Langone Medical Center Insectary). Malaria parasites (4000 sporozoites) were used to infect HepG2 cells (15 000 cells) in a 384-well plate in the presence of compounds **1**–**5** in triplicate. The final concentration of DMSO was 1%, and compounds varied from 0 to 50 μg/mL. Cells were incubated with the compounds for 2 days at 37 °C, and then relative parasite load was determined after addition of Bright-Glo (Promega). Data analysis for HepG2 toxicity and liver-stage malaria activity was carried out using GraphPad Prism, and curves were fit with a standard inhibition dose–response curve to generate an IC_{50} value. All statistical results are the mean IC_{50} value averaged from two independent experiments. Atovaquone was used as a positive control and gave an IC_{50} in blood stage assays of 0.3 nM.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC-MS traces for compounds **1**–**3** and NMR spectra for compounds **1**–**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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